CHARACTERIZATION OF RETINOL BINDING PROTEIN RECEPTOR 2, A PUTATIVE RETINOL TRANSPORTER AND SERUM RETINOL BINDING PROTEIN RECEPTOR

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Vitamin A (retinol) is essential for life; however, little is known about how it is transported into cells. Ninety-five percent of retinol found in blood is bound to Serum Retinol Binding Protein (RBP4), suggesting that RBP4 is important for transport of retinol. Importantly, RBP4 levels are linked to metabolic disorders such as metabolic syndrome and type 2 diabetes. A better understanding of vitamin A transport could provide potential targets for the treatment of these disorders. Our lab recently identified a potential RBP4 receptor protein, Retinol Binding Protein Receptor 2 (RBPR2), also known as Liver Stra6 Homologue (LSH). Cell culture models have revealed that LSH binds RBP4 with high affinity. We hypothesized that mice with a whole body deletion of LSH will show a reduction in retinol and retinyl esters in serum, hepatic tissue and fat.

Our experimental model consisted of transgenic mice with total body deletion of LSH and wild type litter mates, as control. Mice were sacrificed at 7 weeks and tissues stored at -80ºC. Total body deletion of the LSH gene was confirmed by Taqman quantitative PCR. Retinol and retinyl ester levels in liver, fat and serum were assessed using High Performance Liquid Chromatography.

Though no statistically significant differences were found between the retinol or retinyl ester levels in the livers or fat pads of mice with a total body deletion of LSH and wild type mice, there was a significant difference in serum retinol and retinyl ester levels between female knock out mice and female wild type. No significant differences between the two genotypes were observed in the serum of male mice.

Results of this study suggest that LSH is not solely responsible for retinoid transport into cells, or whole body retinol levels, though it may play a subtler role in retinol homeostasis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Vitamin A (retinol) is a fat-soluble micronutrient acquired through diet and involved in the functioning of embryonic development, growth, vision, skin health, the reproductive system, the immune system, and the regulation of over 500 genes. (1, 2) Vitamin A deficiency (VAD) and vitamin A excess (VAE), are known to cause a number of serious health problems, (3) but recently it has been recognized that alterations in proteins involved in vitamin A metabolism can also have serious health consequences - conditions characterized by insulin resistance and glucose intolerance, including type II diabetes. One key characteristic of these conditions is the elevation of Serum Retinol Binding Protein (RBP4), a specific carrier protein for retinol, synthesized in liver and fat. (4) Mouse studies strongly suggest that increased RBP4 in serum may promote insulin resistance, and mice with an RBP4 genetic deletion manifest increased insulin sensitivity. (5) In humans, several studies have shown that an increase in serum RBP4 is associatedwith lowered insulin sensitivity and HDL levels as well as increased body mass index (BMI), triglyceride levels and blood pressure. (6) How RBP4 levels affect insulin sensitivity and whether retinol is directly involved is not known. (7)

Very little is understood about how retinol is transported into cells and a greater understanding would help further our knowledge of how RBP4, and perhaps retinol, are linked to metabolic disease. Since the majority of retinol found in blood is bound to RBP4, it is likely that this protein plays an important role in retinol transport.

Retinol absorption from dietary intake is one retinol transport pathway capable of functioning without RBP4. Animal models with total genetic knock out of RBP4 are viable and have mild symptoms such as night blindness, which resolve when dietary intake of retinol is increased. (1, 8) Retinyl ester (RE), the form of retinol most commonly found in foods, is absorbed in the small intestine (SI) and, as a fat-soluble vitamin, follows the same absorption pathway as other dietary fats that are carried as triglycerides (TG) in chylomicrons. (1)

The exact mechanisms controlling the distribution of retinol outlined above are not known. The high level of regulation observed in vitamin A metabolism suggests there are more targeted ways for retinol to enter cells than through chylomicrons alone. There is evidence of cell surface receptors for RBP4 in a variety of cell types and tissues, including epithelial cells in the intestine. (9, 10) One such cell surface receptor and retinol transport protein, Stimulated by Retinoic Acid Gene 6 (STRA6), appears to act as a retinol channel or transporter and exhibits high binding affinity with RBP4. (11) It is highly expressed in many tissues during embryonic development and is found in adult retina, brain, spleen, kidney, female genital tract and testis, but is *not* known to be expressed in liver or fat, metabolically active tissues. (11)

Because STRA6 is not significantly expressed in tissues targeted by insulin, it is unlikely to play a direct role in those tissues in disease states characterized by insulin resistance, in spite of its high binding affinity with RBP4. However, our lab recently identified a potential RBP4 receptor protein, Retinol Binding Protein Receptor 2 (RBPR2), also called Liver Stra6 Homologue (LSH), which *is* expressed in fat and liver (as well as intestine); tissues that play a role in insulin resistance. Regions of the LSH

sequence are highly conserved with STRA6, most importantly, the region analogous to the predicted STRA6-RBP4 binding site.

We propose that LSH could be the retinol transporter involved in regulating the movement of Vitamin A across cell membranes into metabolically significant tissues, suggesting it may play a role in retinol homeostasis in diseases characterized by insulin resistance. To date, LSH has been studied only in cell culture models. Recently obtained *in vitro* data from our lab show that over-expression of this protein causes increased retinol transport and increased RBP4 binding. It is possible that LSH may play a crucial role in whole body retinol metabolism but little or nothing is known about the role of LSH *in vivo*.

We hypothesize that LSH is a membrane transport protein for retinol, and as such, contributes to the movement of retinol and RE across cell membranes. To test this hypothesis, we have used an *in vivo* transgenic mouse model with a total body deletion of the LSH protein and compared it to age-matched wild type littermates, focusing on the tissues known to store or transport retinol: liver, fat and serum. Retinol and retinyl ester content of these tissues was measured by high performance liquid chromatography (HPLC). A phenotype of reduced retinol levels in the transgenic model would further support the *in vitro* data suggesting LSH is necessary for transport of retinol.

METHODS

Animals and Diets

The animal model used for this project is derived from the C57-black mouse strain. It is a knock out mouse developed as part of a trans-NIH Knock Out Mouse initiative, (KOMP), a project that utilizes high-throughput technology to engineer mouse embryonic stem cells containing null mutations as a public resource. (12) In this model there is a disruption in exon 6 of the LSH gene and it is predicted to produce unstable mRNA, incapable of being translated into a functional protein. If mRNA remains stable and is transcribed in spite of the presence of the disruption, it is likely that any proteins translated would be truncated and nonfunctional. Preliminary data from gene expression studies in our lab indicate that these mice have confirmed genetic KO of LSH mRNA in liver, though further confirmation of the KO by measuring translated protein levels with western blot is still in progress. We call this mouse model Liver Stra6 Homologue flox-beta-geo (*LSH flox-ß geo)*.

Samples used for this work were taken from three cohorts of mice: Cohort 1: Mixed age, mixed gender homozygous (HOM) LSH flox ß-geo mice and their wild type (WT) and heterozygous (HET) littermates; Cohort 2: C57Bl wild type (WT) adult male mice; and Cohort 3: 7-week-old mixed gender HOM LSH flox ß-geo mice and their WT littermates. The HOM mice have two copies of the "knockout allele" (and no WT allele), expressed in all of their tissues; HET mice have one copy of the knockout allele and one WT allele. (See Tables 1, 2 and 3.)

Table 1: Cohort 1, Adult mice

ID	SEX	AGE	LINE	Genotype
4291	M	35w	LSH-Flox-ß-geo	wt/wt
4292	M	35w	LSH-Flox-ß-geo	wt/wt
4293	F	35w	LSH-Flox-ß-geo	fbg/wt
4294	F	35w	LSH-Flox-ß-geo	fbg/wt
4295	F	35w	LSH-Flox-ß-geo	fbg/wt
4297	F	35w	LSH-Flox-ß-geo	fbg/wt
4298	F	35w	LSH-Flox-ß-geo	fbg/fbg
4316	M	28w 6d	LSH-Flox-ß-geo	fbg/wt
4317	M	28w 6d	LSH-Flox-ß-geo	wt/wt
4318	M	28w 6d	LSH-Flox-ß-geo	fbg/wt
4320	F	28w 6d	LSH-Flox-ß-geo	fbg/fbg
4322	M	28w 3d	LSH-Flox-ß-geo	fbg/fbg
4324	M	28w 3d	LSH-Flox-ß-geo	fbg/fbg
4325	M	28w 3d	LSH-Flox-ß-geo	fbg/wt
4326	M	28w 3d	LSH-Flox-ß-geo	fbg/fbg
4351	M	20w 6d	LSH-Flox-ß-geo	fbg/fbg
4248	F	37w	LSH-Flox-ß-geo	wt/wt
4353	M	9w 2d	LSH-Flox-ß-geo	wt/wt
4354	M	9w 2d	LSH-Flox-ß-geo	wt/wt

Cohort 1: 12 males, 8 females, 6 WT, 6 HOM, 8 HET

Table 2: Cohort 2, WT mice

ID	Sex	Age	Line	Genotype
3023	M	26 w	C57Bl	wt
3032	M	20w 6d	C57Bl	wt
3033	M	20w 6d	C57Bl	wt
3034	M	20w 6d	C57Bl	wt
3035	M	20w 6d	C57Bl	wt
3036	M	20w 6d	C57Bl	wt
3037	M	20w 6d	C57Bl	wt
3038	M	20w 6d	C57Bl	wt

Table 3: Cohort 3, 7-week-old-mice

ID	SEX	AGE	Mouse Line	Genotype
4092	M	7w	LSH-Flox-ß-geo	fbg/fbg
4094	M	7w	LSH-Flox-ß-geo	wt/wt
4096	M	7w	LSH-Flox-ß-geo	fbg/fbg
4099	M	6w 6d	LSH-Flox-ß-geo	wt/wt
4100	M	6w 6d	LSH-Flox-ß-geo	wt/wt
4676	F	7w	LSH-Flox-ß-geo	wt/wt
4693	M	7w	LSH-Flox-ß-geo	fbg/fbg
4694	M	7w	LSH-Flox-ß-geo	fbg/fbg
4695	F	7w	LSH-Flox-ß-geo	fbg/fbg
4698	F	7w	LSH-Flox-ß-geo	fbg/fbg
4699	M	7w	LSH-Flox-ß-geo	fbg/fbg
4702	F	7w	LSH-Flox-ß-geo	fbg/fbg
4704	F	6w 6d	LSH-Flox-ß-geo	wt/wt
4705	M	7w	LSH-Flox-ß-geo	wt/wt
4707	M	7w	LSH-Flox-ß-geo	wt/wt
4708	M	7w	LSH-Flox-ß-geo	fbg/fbg
4709	M	7w	LSH-Flox-ß-geo	wt/wt
4713	F	7w	LSH-Flox-ß-geo	wt/wt
4714	F	7w	LSH-Flox-ß-geo	fbg/fbg
4715	F	7w	LSH-Flox-ß-geo	fbg/fbg
4722	F	6w 6d	LSH-Flox-ß-geo	wt/wt
4914	M	7w 1d	LSH-Flox-ß-geo	fbg/fbg
4915	M	7w 1d	LSH-Flox-ß-geo	fbg/fbg

Cohort 3: 14 males, 9 females; 13 HOM, 10 WT

All animals in the study were fed a standard chow diet consisting of 37 IU/g vitamin A (One IU is equal to 0.3 ug of retinol, 0.55 g retinyl palmitate or 0.6 ug of b-carotene) [Research Diets Inc]. (13) Animals were fasted for 16 hours prior to sacrifice, their tissues harvested and immediately frozen in liquid nitrogen, and then stored at -80º until the time of analysis.

In the first cohort of mixed-age, mixed-gender mice (Table 1), liver and serum were analyzed to determine retinoid content using HPLC. Retinoid levels from HOM mice were compared to WT and (HET) littermates. In the second cohort of eight

5-to7- month-old male WT C57-black mice (Table 2), liver was analyzed for retinol and RE content, then these levels compared to LSH expression levels measured by Quantitative PCR. Male mice were used for this control experiment to eliminate the possibility of sample variation likely to occur with older female mice due to loss of RE stores during pregnancy and lactation.

In the third cohort of mixed gender, 7-week-old mice (Table 3), liver, fat and serum were analyzed for retinoid content and levels of HOM mice compared to WT. LSH expression levels were also measured by Quantitative PCR to confirm genetic deletion of LSH mRNA.

Analysis of Retinoids in Tissue

The preparation and analysis of animal tissues used for this study was similar to methods and protocols widely practiced in the study of retinoids and other fat-soluble vitamins. (14) All preparation was conducted under orange/yellow filtered light to avoid isomerization of photosensitive retinoids contained in the samples and standard. Only glass tubes and pipettes were used for retinoid extraction and storage of the purified samples.

Sample Preparation

Whole livers from each animal were pulverized to a fine powder while frozen with a ceramic pestle over liquid nitrogen and mixed thoroughly to ensure homogeneity of the final weighed sample. Samples from fat were sliced while frozen and weighed. Tissue and serum from each mouse was weighed/measured and prepared in duplicate. Twenty to 40 mg of tissue were placed in 2 ml screw-top tubes [Fisher Scientific] containing 2 mm ceramic beads [BioSpec Products] along with 500 µl PBS and 500 µl

butylated hydroxy toluene (BHT) in ethanol [MP Biomedicals]. Contents of tubes were then homogenized in a Qiagen Tissuelyser.

Serum was prepared by mixing 30 µl with 500 µl PBS and 500 µl BHT and vortexed for 3-4 seconds to mix.

Internal Standard (IS)

After mixing/homogenizing, 10 µl of 2mM retinyl acetate in ethanol vehicle were added to each sample as an internal standard. After addition of the standard, samples were transferred to a 15 ml screw-top glass culture tube [Kimble Chase]. Proteins were precipitated with the addition of 1 ml 0.025M potassium hydroxide in ethanol and vortexed for 3 seconds. Ten ml hexane [Sigma Aldrich] were immediately added to the homogenate and tubes were capped and vortexed 10 seconds to mix. The samples were centrifuged for 3 minutes at 1000 rpm, after which the organic layer was transferred to clean glass tubes and evaporated under nitrogen until dry [Nitrovap]. Dried samples were resuspended in 600 µl acetonitrile and either analyzed immediately by HPLC or stored for no more than 2 days at -20º in amber glass vials [Fisher Scientific].

HPLC Analysis of Retinol and Retinyl Ester

One hundred µl of the purified sample were injected onto a Zorbax C18 reverse phase column [Agilent] using an Agilent 1100 HPLC system with UV diode array detector, absorbance 325 nm. (14, 15) An additional, separate injection of 10 µl was performed on liver samples so that the RE signal was within the linear detection range. Liver is the primary storage site for retinyl esters and the levels can be high, so a smaller injection is necessary to get an accurate reading. Analytes were separated at 1 ml/minute with 11% H_2O/89% acetonitrile/ 0.1% formic acid for 9 minutes, followed by a linear

gradient over 2 minutes to 100% acetonitrile. 100% acetonitrile was maintained for 2 minutes, followed by a linear gradient over 2 minutes to 5% acetonitrile/1, 2-dichloroethane. Five percent acetonitrile/1, 2-dichloroethane was maintained for 2 minutes, followed by a linear gradient over 2 minutes back to 100% acetonitrile, which was maintained for 2 minutes, then followed by a linear gradient over 2 minutes back to initial conditions. (15) Retention times for analytes were retinol at 4.8 – 5.2 min, retinyl acetate (IS) at 8.9 – 9.4 minutes, and RE at 16.2 -17.4 minutes. Total liver retinyl esters are composed of several isomers, differing only in their fatty acid moiety. Peak areas were used for comparison and the multiple peaks that represent retinyl esters were added together to give the total retinyl ester.

Peak areas for RE were corrected for the peak area of the IS and then divided by the weight of the tissue used to make the purified sample. Peak areas for ROH were divided by a value representing the average value of the IS peaks (Tables 4 - 7).

Gene Expression

Expression of LSH in WT and HOM mouse liver was determined using Taqman quantitative PCR. [Applied Biosystems, Roche]

Statistical Analysis

A Pearson product-moment correlation coefficient was calculated to analyze the relationship between tissue retinoid levels and LSH gene expression levels. Differences in retinoid levels between the HOM and WT mice were tested by mean value T-test. A p-value of $\leq .05$ was considered significant.

Table 4: Liver Retinol (ROH) and Retinyl Esters (RE) in Cohort 1

ID/Genotype	SEX	AGE	ROH (U/gm)	RE (U/gm)
WT54	M	9w 2d	1.193156867	112.4164391
WT53	M	9w 2d	1.531141409	117.8163134
WT91	M	35w	0.64318932	306.6439414
WT92	M	35w	0.810860071	215.6270112
WT48	F	37w	1.040742222	334.5890707
WT17	M	28w 6d	1.141513986	317.1348076
HET16	M	28w 6d	0.757673193	291.1202308
HET94	F	35w	0.739676121	219.5821734
HET93	F	35w	0.832083825	262.0370437
HET18	M	28w 6d	0.898150653	218.1135471
HET25	M	28w 3d	1.396629801	317.7467341
HET95	F	35w	1.408608696	255.2103441
HET97	F	35w	1.39347366	307.7406755
HOM98	F	35w	1.09296284	342.9480701
HOM26	M	28w 3d	0.90434188	245.9362885
HOM51	M	20w 6d	1.909334574	337.9983939
HOM20	F	28w 6d	1.185084148	357.2046381
HOM22	M	28w 3d	1.216540642	258.3608708
HOM24	M	28w 3d	1.021960937	307.3442322

Table 5: Retinol (ROH) and Retinyl Esters (RE) measured by HPLC in serum, Cohort 1

ID/Genotype	SEX	AGE	ROH (U/gm)	RE (U/gm)
WT 54	M	9w 2d	0.0011873	112.4164391
WT53	M	9w 2d	0.000933095	117.8163134
WT92	M	35w	0.00080506	215.6270112
WT48	F	37w	0.001150301	334.5890707
WT17	M	28w 6d	0.001095808	317.1348076
HOM98	F	35w	0.001129813	342.9480701
HOM26	M	28w 3d	0.000887245	245.9362885
HOM51	M	20w 6d	0.001769956	337.9983939
HOM20	F	28w 6d	0.00108079	357.2046381
HOM22	M	28w 6d	0.001693445	258.3608708
HOM24	M	28w 3d	0.000893196	307.3442322

Table 6: Retinol (ROH) and Retinyl Esters (RE) measured by HPLC in liver and fat of Cohort 3.

ID/Genotype	SEX	Liver ROH (U/gm)	Liver RE (U/gm)	Fat ROH (U/gm)	Fat RE (U/gm)
4676 - WT	F	0.877669482	119.1320577	1.646588821	1.413878023
4695 – HOM	F	0.929658308	126.2356129	1.767851647	1.502988887
4698 – HOM	F	1.019013773	112.2225454	1.707967072	1.826110479
4702 – HOM	F	0.930480495	94.52404575	1.829911136	2.499088329
4704 – WT	F	1.081317356	110.9609463	1.728569959	1.296840534
4713 – WT	F	0.716349535	131.5947178	1.792467079	1.094253883
4714 – HOM	F	0.889841603	155.8375025	1.555592538	0.813154602
4715 – HOM	F	1.588855493	210.8734368	1.778630133	0.981035783
4722 – WT	F	0.936116085	114.7072437	2.06411385	0.758790332
4092 – HOM	M	1.084587225	174.219782	1.972172515	1.273242346
4094 – WT	M	1.095386382	184.67234	1.682185932	1.158441362
4096 – HOM	M	1.293241156	173.8329611	2.019003573	1.063857635
4099 – WT	M	1.222623474	211.2052059	2.111866945	1.309998599
4100 – WT	M	1.405901314	168.2190438	1.963332649	1.026814708
4693 – HOM	M	1.121641637	98.18710663	2.254686494	1.111249684
4694 – HOM	M	1.227914894	96.43866492	1.889333853	1.081642148
4699 – HOM	M	1.423769748	139.8968805	1.848506356	0.9976
4705 – WT	M	0.816140399	79.81064087	1.819841103	1.172879406
4707 – WT	M	0.772800634	135.7390432	1.164991678	1.129608177
4708 – HOM	M	0.805806278	115.9110289	1.632136731	1.001621593
4709 –WT	M	0.73269752	119.7490966	1.925346018	1.10996065
4914 – HOM	M	1.253703115	189.8921485	2.104546186	1.132836053
4915 - HOM	M	1.429785897	254.1	2.226198463	1.446050636

Table 7: Retinol (ROH) and Retinyl Esters (RE) measured by HPLC in serum of Cohort 3.

ID/Genotype	SEX	Liver ROH (U/µl)	Liver RE (U/µl)
4676 - WT	F	0.002085024	0.000153472
4695 – HOM	F	0.00206342	0.000154611
4698 – HOM	F	0.002040164	0.000167552
4702 – HOM	F	0.002008889	0.000175247
4704 – WT	F	0.002064276	0.000130884
4713 – WT	F	0.002016156	0.000139764
4714 – HOM	F	0.002086514	0.00049851
4715 – HOM	F	0.001979697	0.000211482
4722 – WT	F	0.002159136	0.000337779
4092 – HOM	M	0.00217126	0.000274746
4094 – WT	M	0.002134909	0.000258127
4096 – HOM	M	0.002167625	0.000202035
4099 – WT	M	0.00213636	0.000217753
4100 – WT	M	0.002157416	0.000262465
4693 – HOM	M	0.002227048	0.000173913
4694 – HOM	M	0.002244193	0.000154169
4699 – HOM	M	0.002010967	0.000155085
4705 – WT	M	0.002056826	0.000180747
4707 – WT	M	0.00209701	0.000164206
4708 – HOM	M	0.002072041	0.000407125
4709 –WT	M	0.002130936	0.000138286
4914 – HOM	M	0.002222861	0.000497139
4915 - HOM	M	0.002315972	0.0002111

RESULTS

In the first cohort of transgenic mice of mixed age and gender (Tables 1 and 4), the RE levels in liver and serum of young WT mice (~2-month-old) were found to be significantly lower than older mice of both genotypes. There were no significant differences in the ROH levels between any of the groups, and no other differences were observed between RE levels of HOM and WT mice (Figures 1 – 6).

In the analysis of liver from the second cohort of adult male C57Bl mice, (Table 2), retinoid levels were found to have a strong *negative* association with LSH mRNA levels in liver (Figure 7).

In the analysis of liver from the third cohort of 7-week-old WT mice (Tables 3 and 5), no correlation was found between retinoids and LSH mRNA levels (Figure 8).

Quantitative PCR taqman analysis of the livers from mice in the 7-week-old cohort suggests complete KO of LSH mRNA in mice with homozygous genotype compared to WT and HET littermates (Figure 9).

Seven-Week-Old Cohort, Liver

HPLC analysis of livers from mice in the 7 week old cohort showed no significant differences in RE or ROH levels between HOM and WT (Figures 10 and 11). No trends or differences were detected when groups were divided by gender (data not shown).

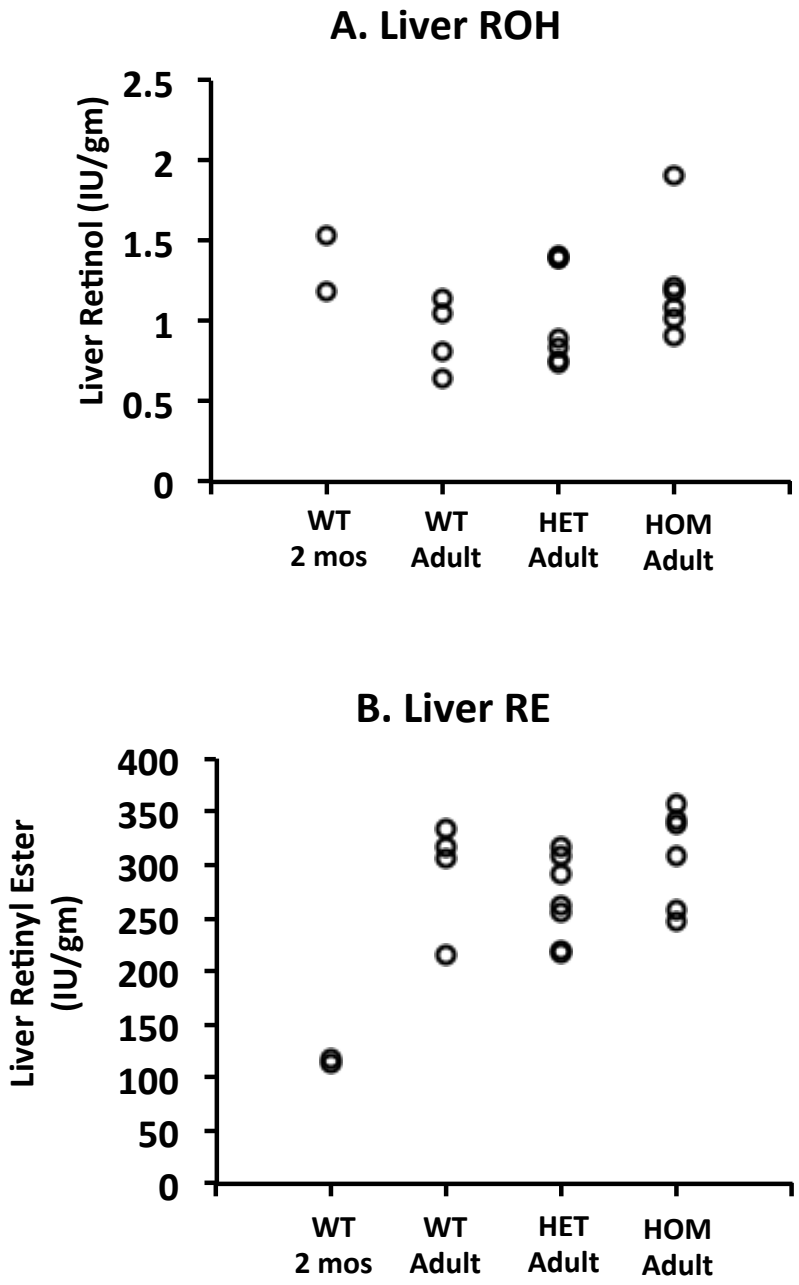


Figure 1: Mice of mixed genotype, age and gender: liver retinol and retinyl ester. Shows range of retinoids for each of the experimental groups. Each data point represents a separate mouse. WT-wild type, HET – heterozygous, HOM – homozygous. A. Retinol levels measured in livers of young WT, adult WT, adult HET and adult HOM mice. B. Retinyl esters measured in livers of young WT, adult WT, adult HET and adult HOM mice. (adult mice: >6 mos)

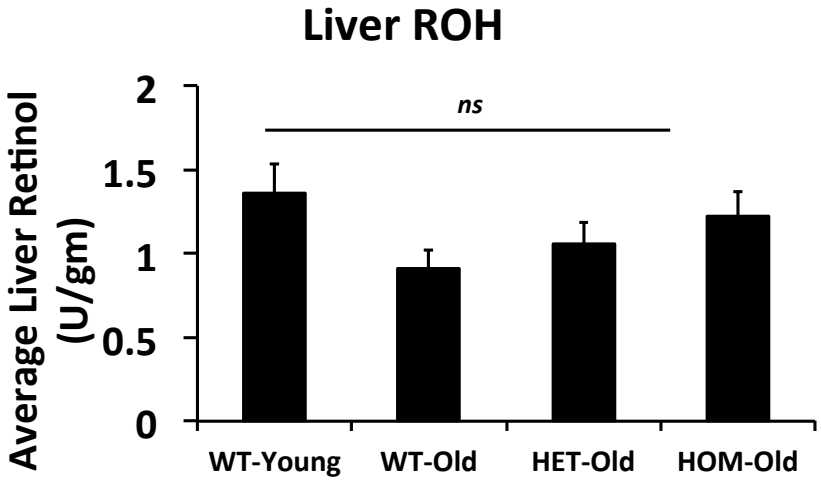


Figure 2: Mice of mixed genotype, age and gender: average liver retinol. Retinol levels measured in livers of young WT, old WT, old HET and old HOM mice.

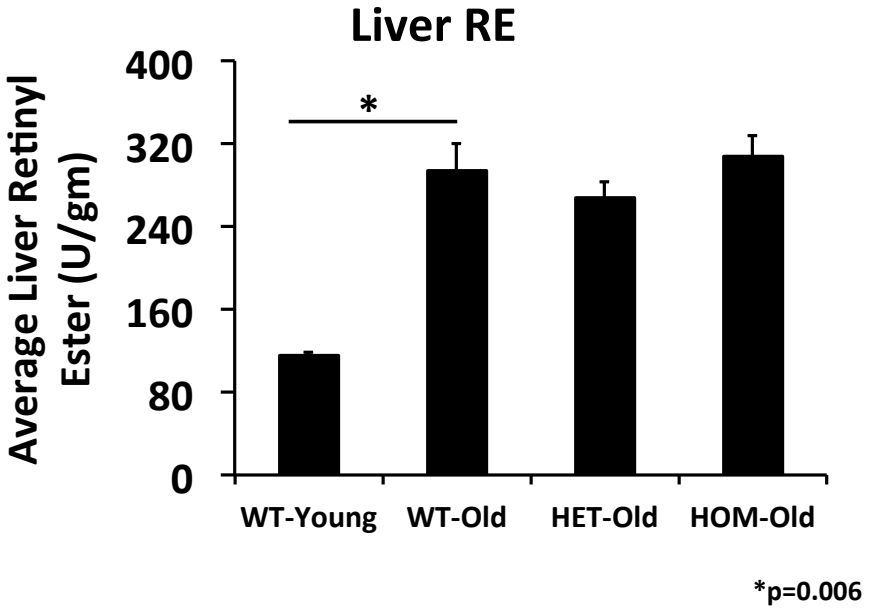


Figure 3: Mice of mixed genotype, age and gender: average liver retinyl ester. Levels measured in livers of young WT, old WT, old HET and old HOM mice.

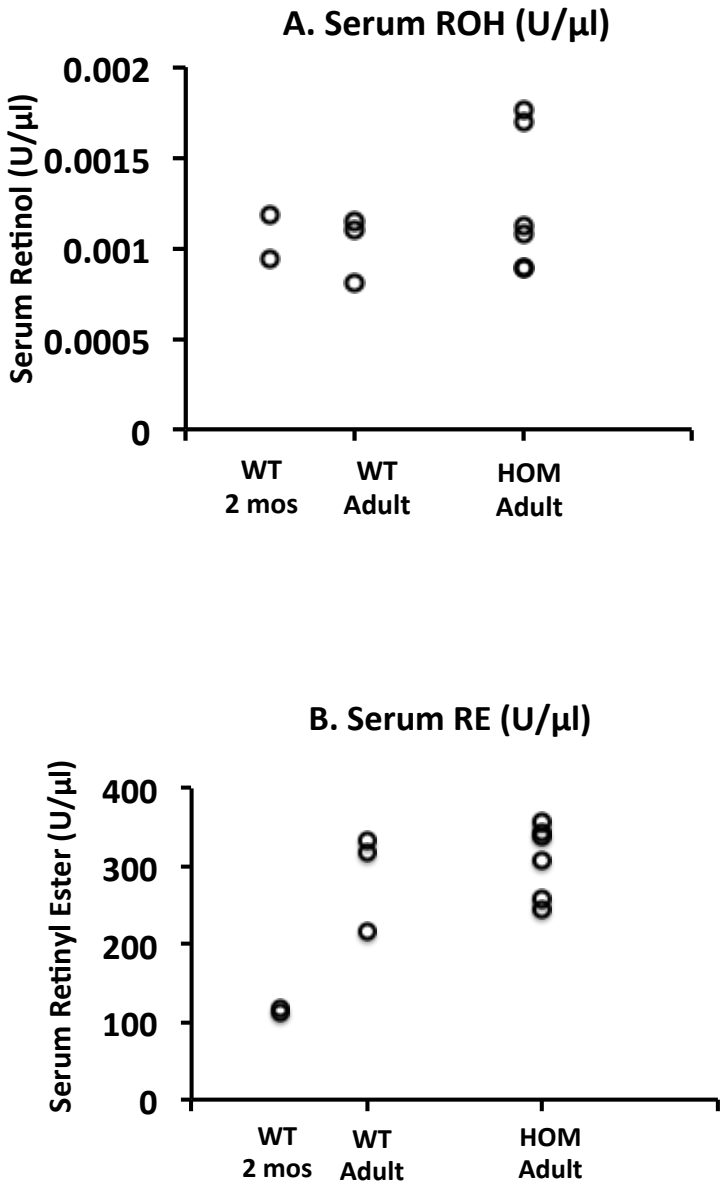


Figure 4: Mice of mixed genotype, age and gender: serum retinol and retinyl ester. A. Retinol levels measured in serum of young WT, adult WT, adult HET and adult HOM mice. B. Retinyl esters measured in serum of young WT, adult WT, adult HET and adult HOM mice. (adult mice: >6 mos)

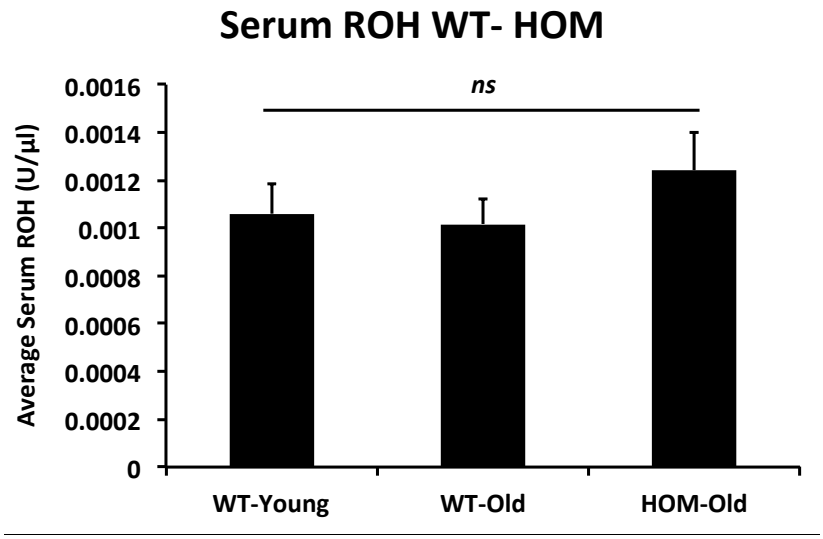


Figure 5: Mice of mixed genotype, age and gender: average serum ROH. Average retinol levels measured in serum of young WT, old WT, and old HOM mice.

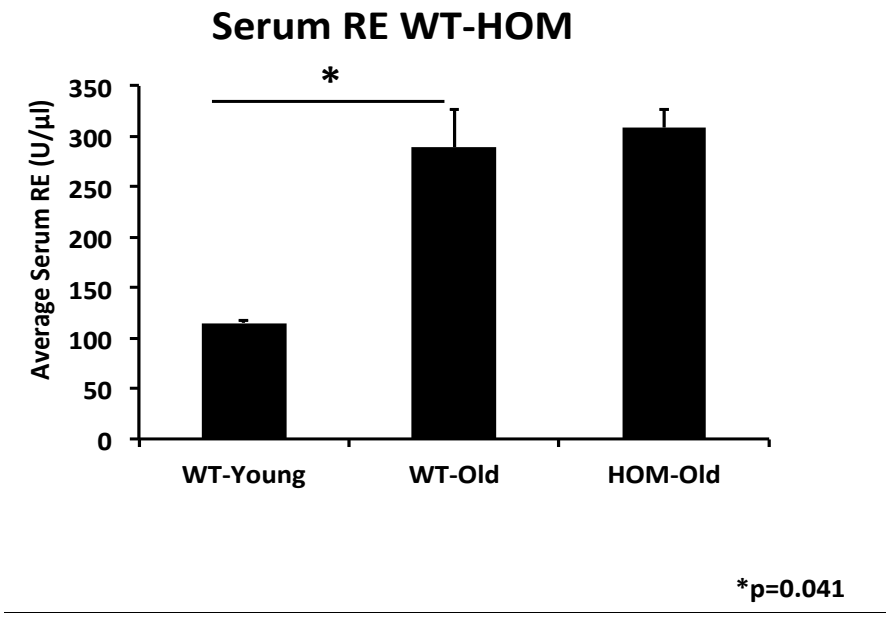


Figure 6: Mice of mixed genotype, age and gender: average serum RE. Average retinyl ester levels measured in serum of young WT, old WT, and old HOM mice.

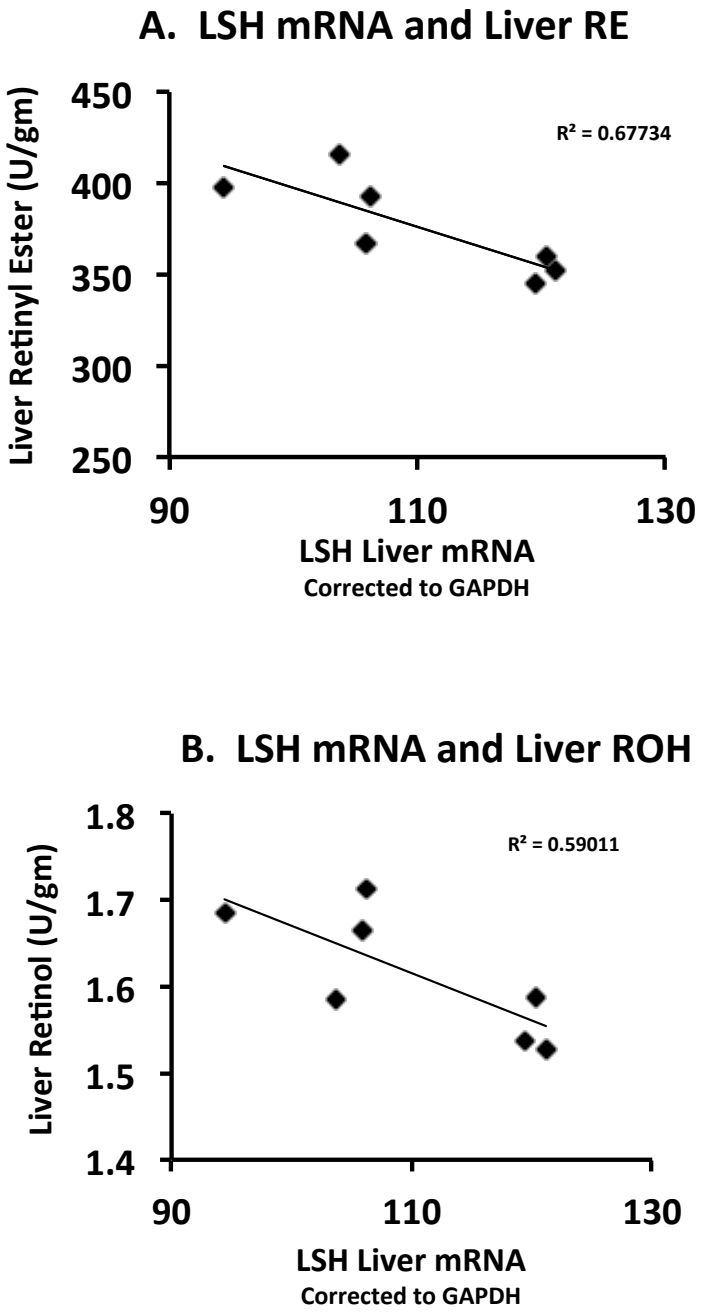
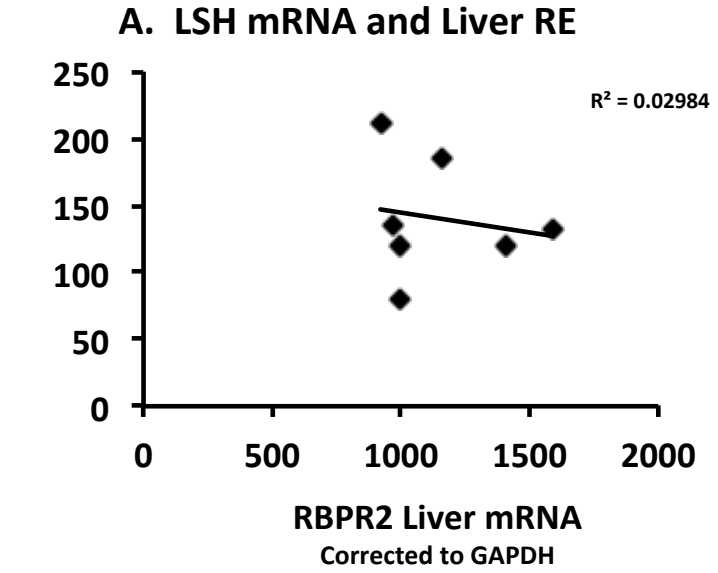


Figure 7: Adult male WT mice: LSH mRNA with liver RE and ROH. A. Liver retinyl ester levels compared to LSH gene expression in liver of adult male WT mice. B. Liver retinol levels compared to LSH gene expression in liver of adult male WT mice.



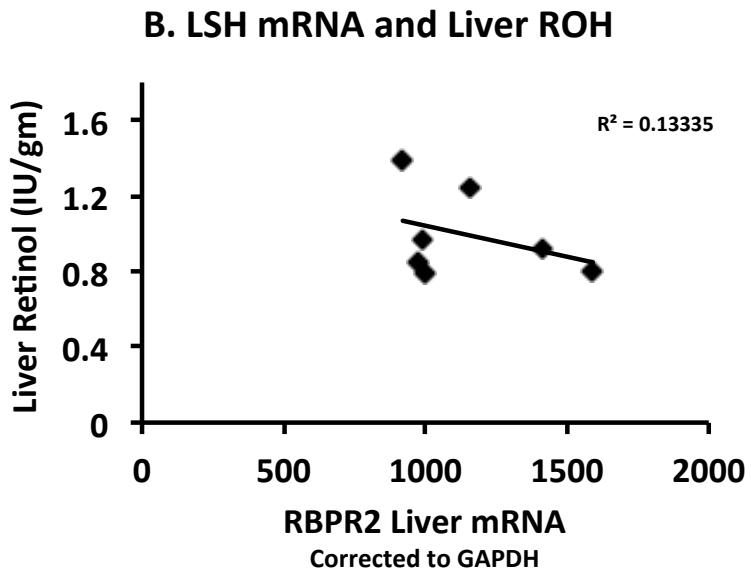


Figure 8: 7-week-old male WT mice: LSH mRNA with Liver RE and ROH. A. Liver retinyl ester levels compared to LSH gene expression in liver. B. Liver retinol levels compared to LSH gene expression in liver. Neither correlation is as strong as in measurements taken from WT control (Figure 7).

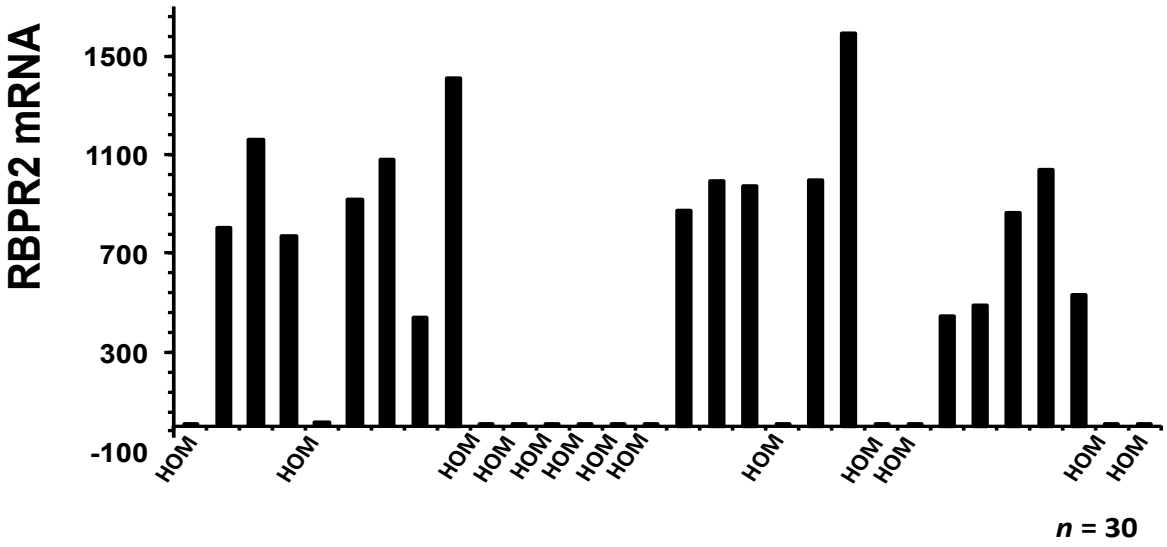


Figure 9: Confirmed LSH knock-out in LSH Flox ß-geo mouse liver. Taqman assay corrected for GAPDH. LSH mRNA levels of liver samples from each of the mice in the 7 week old cohort; WT, HET and HOM – homozygous (HOM) mice show extremely low levels of LSH mRNA in liver.

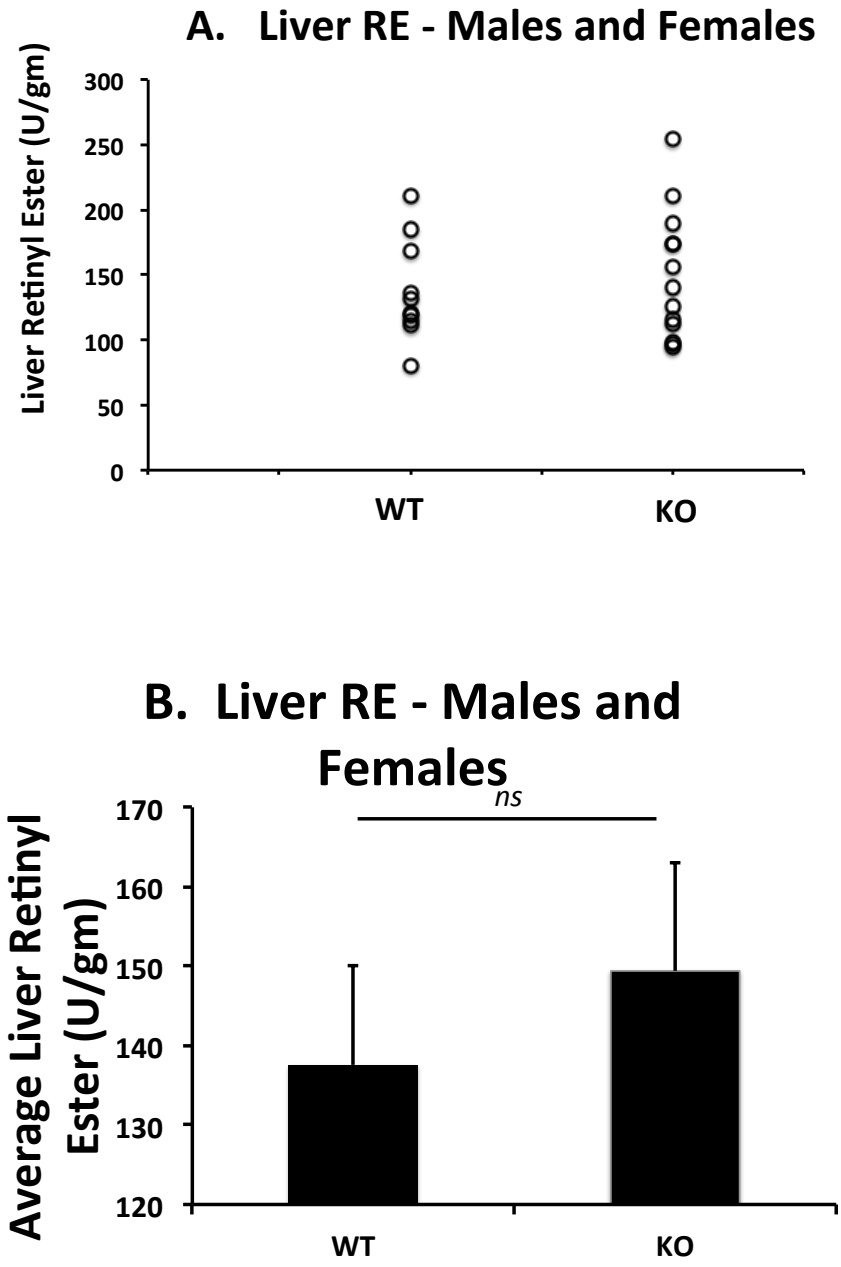


Figure 10: 7-week-old mice: liver RE. A. Retinyl ester levels measured in liver of WT and HOM male and female mice. B. Average retinyl ester levels measured in liver of WT and HOM male and female mice.

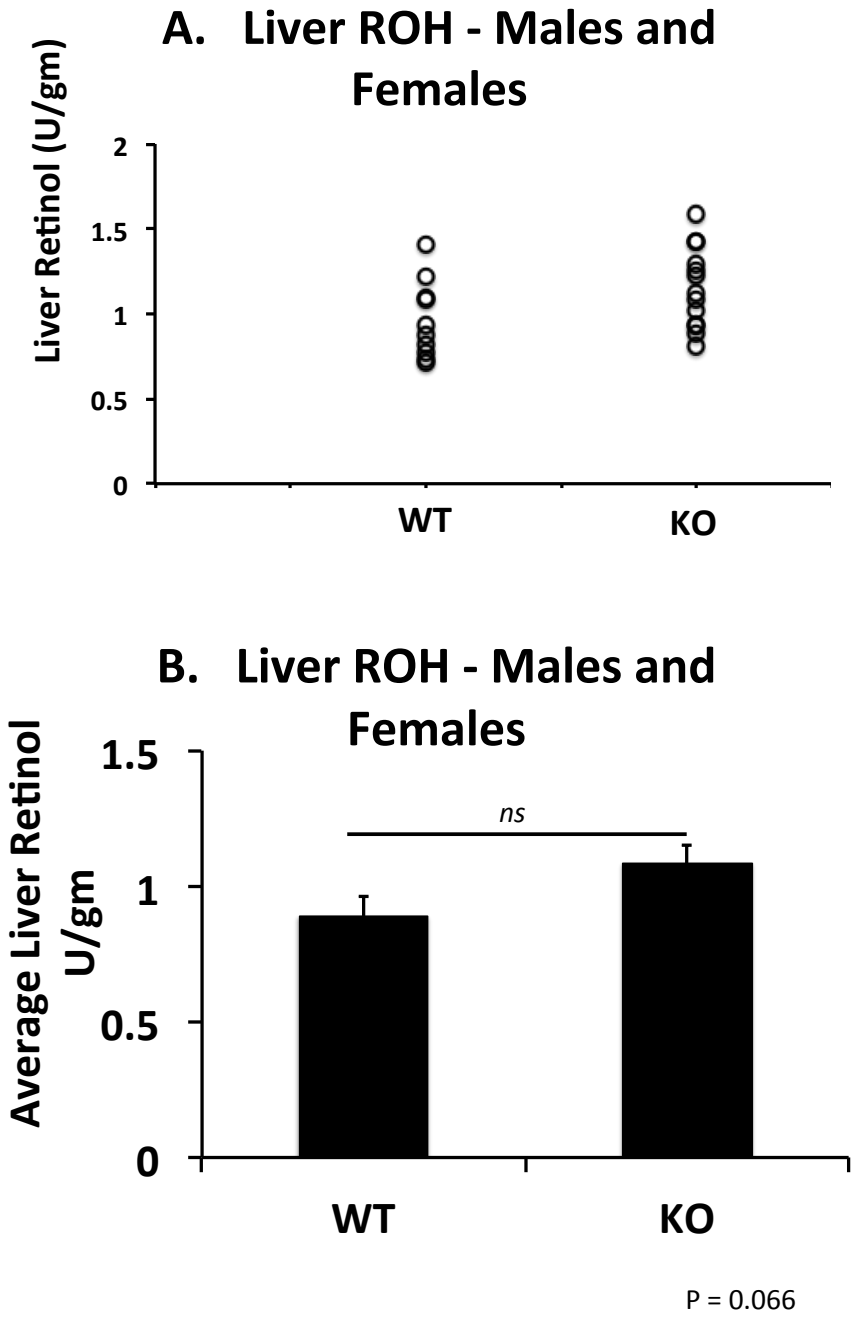


Figure 11: 7-week-old mice: liver ROH-males and females. A. Retinol levels measured in liver of WT and HOM male and female mice. B. Average retinol levels measured in liver of WT and HOM male and female mice.

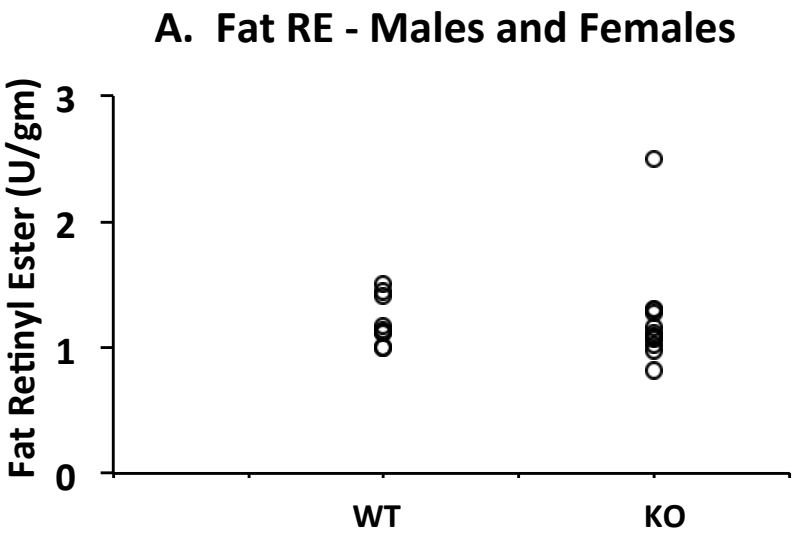
Seven-Week-Old Cohort; Fat

HPLC analysis of fat from mice in the 7-week-old cohort showed no significant differences in RE or ROH levels between HOM and WT (Figures 12 and 13) No trends or differences were detected when groups were divided by gender (data not shown).

Seven-Week-Old Cohort – Serum

HPLC analysis of serum in this cohort showed no significant differences in RE or ROH levels between HOM and WT (Figures 14 and 15). However, when divided into gender-specific groups, the serum of female mice showed a significant difference in RE levels between HOM and WT (Figure 16)

HPLC analysis of serum of female mice also showed a significant difference in ROH levels between HOM and WT (Figure 17). Further analysis of serum from three females from the first adult cohort of LSH flox ß-geo mice also shows what appears to be a significant difference in ROH and RE levels between HOM and WT (Figure 18), though the number of samples is too small for statistical analysis.



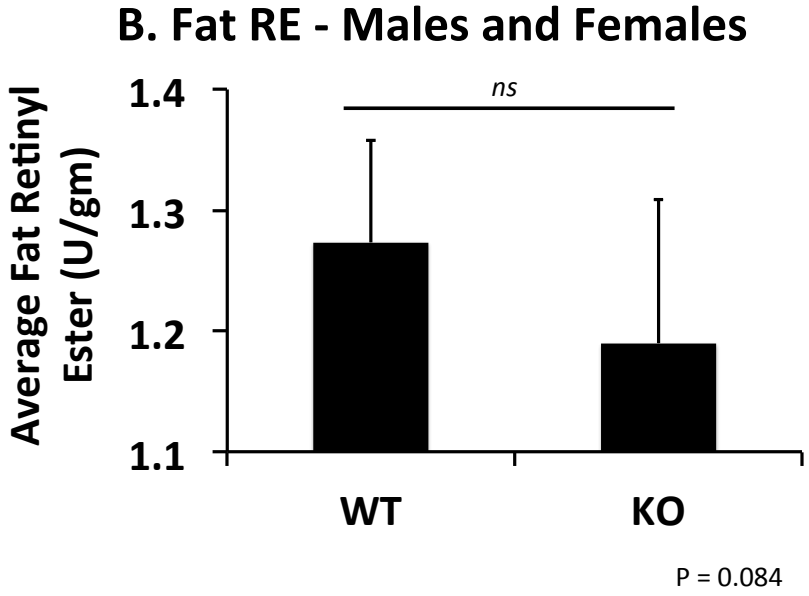


Figure 12: 7-week-old mice: fat RE – males and females. A. Retinyl ester levels measured in fat of WT and HOM male and female mice. B. Average retinyl ester levels measured in fat of WT and HOM male and female mice.

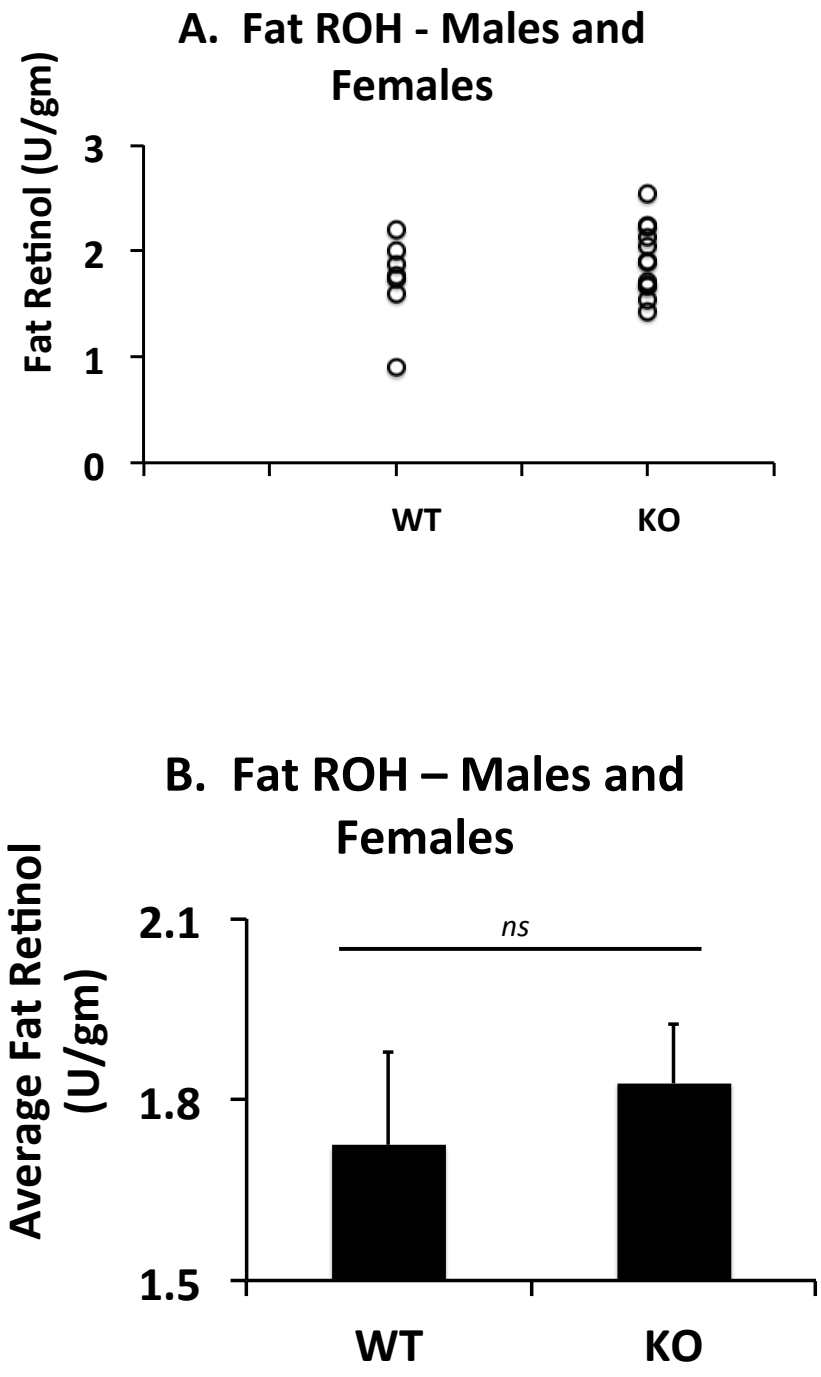


Figure 13: 7-week-old mice: fat ROH – males and females. A. Retinol levels measured in fat of WT and HOM male and female mice. B. Average retinol levels measured in fat of WT and HOM male and female mice.

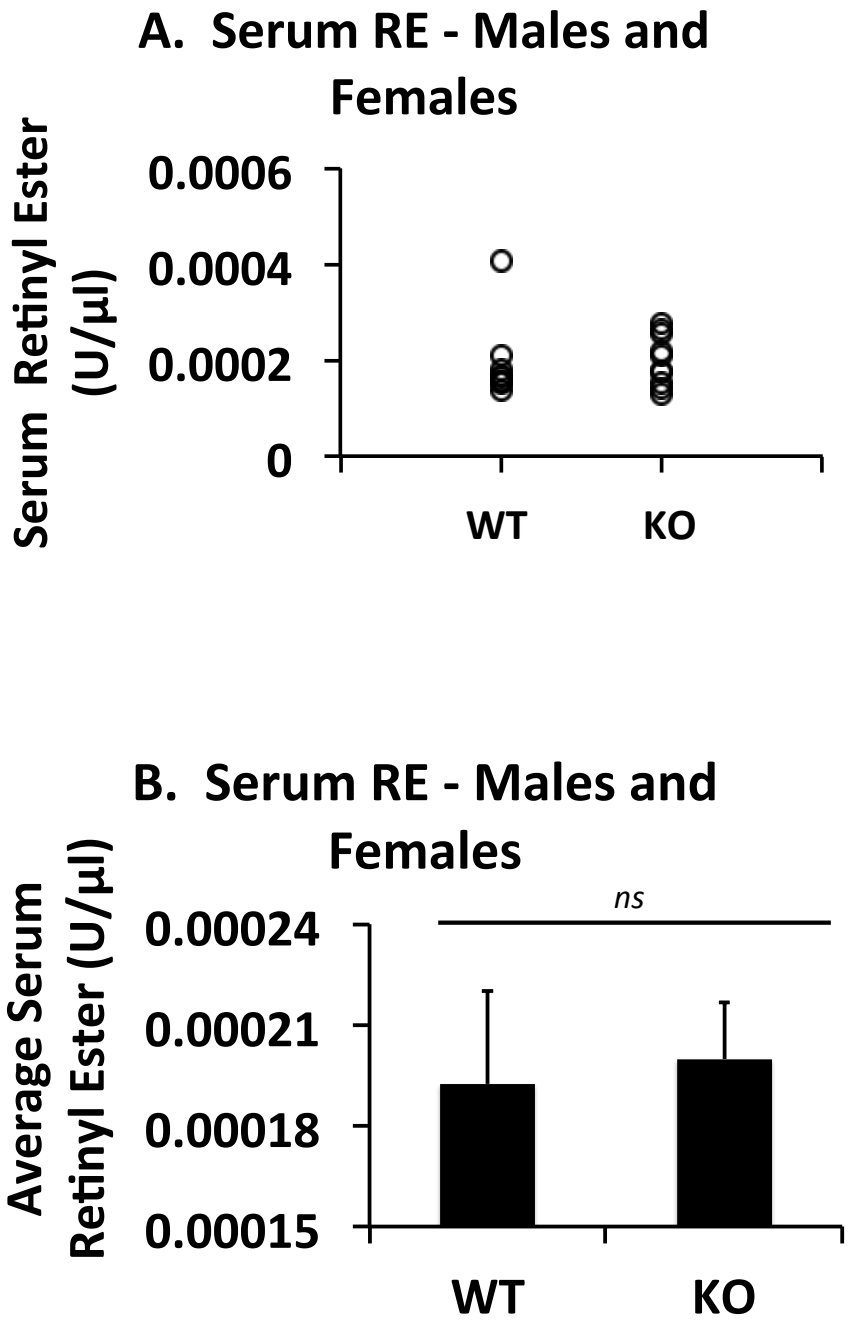


Figure 14: 7-week-old mice: serum RE – males and females. A. Retinyl ester levels measured in serum of WT and HOM male and female mice. B. Average retinyl ester levels measured in serum of WT and HOM male and female mice.

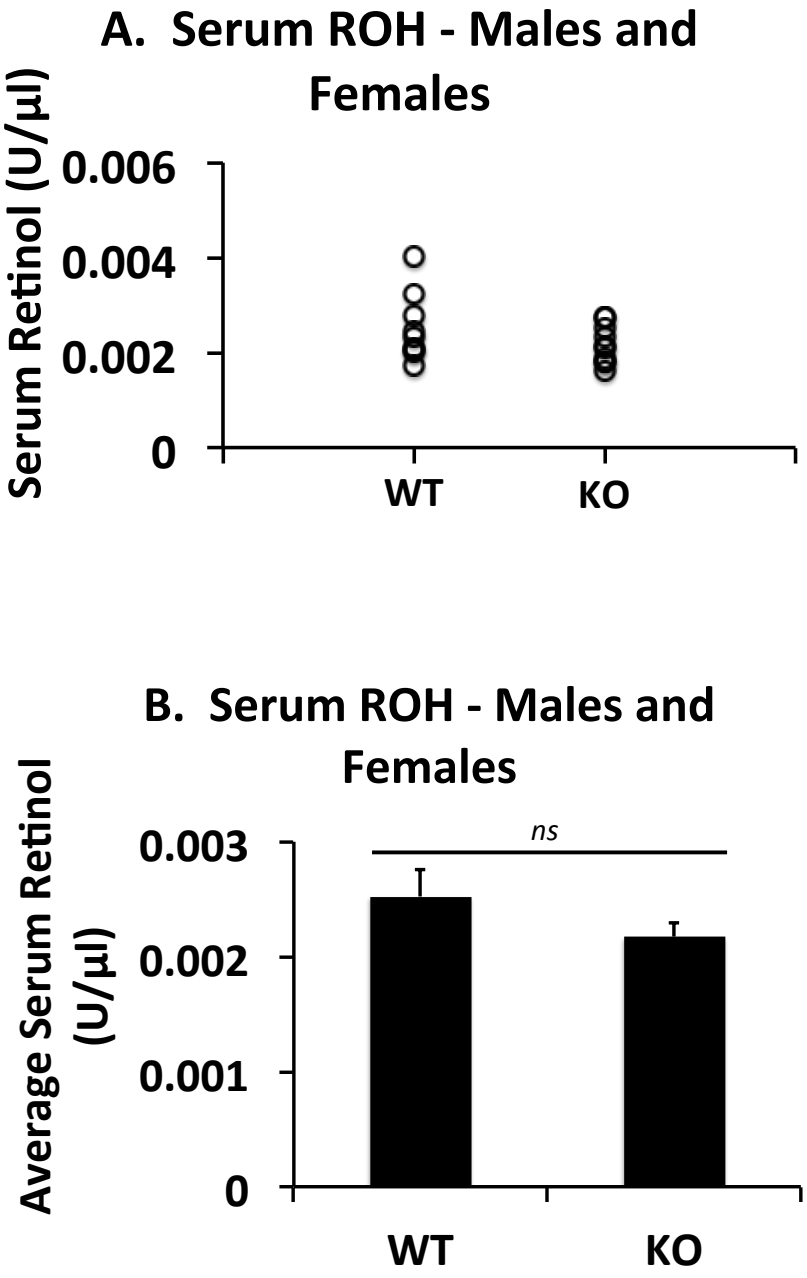


Figure 15: 7-week-old mice: serum ROH – males and females. A. Retinol levels measured in serum of WT and HOM male and female mice. B. Average retinol levels measured in serum of WT and HOM male and female mice.

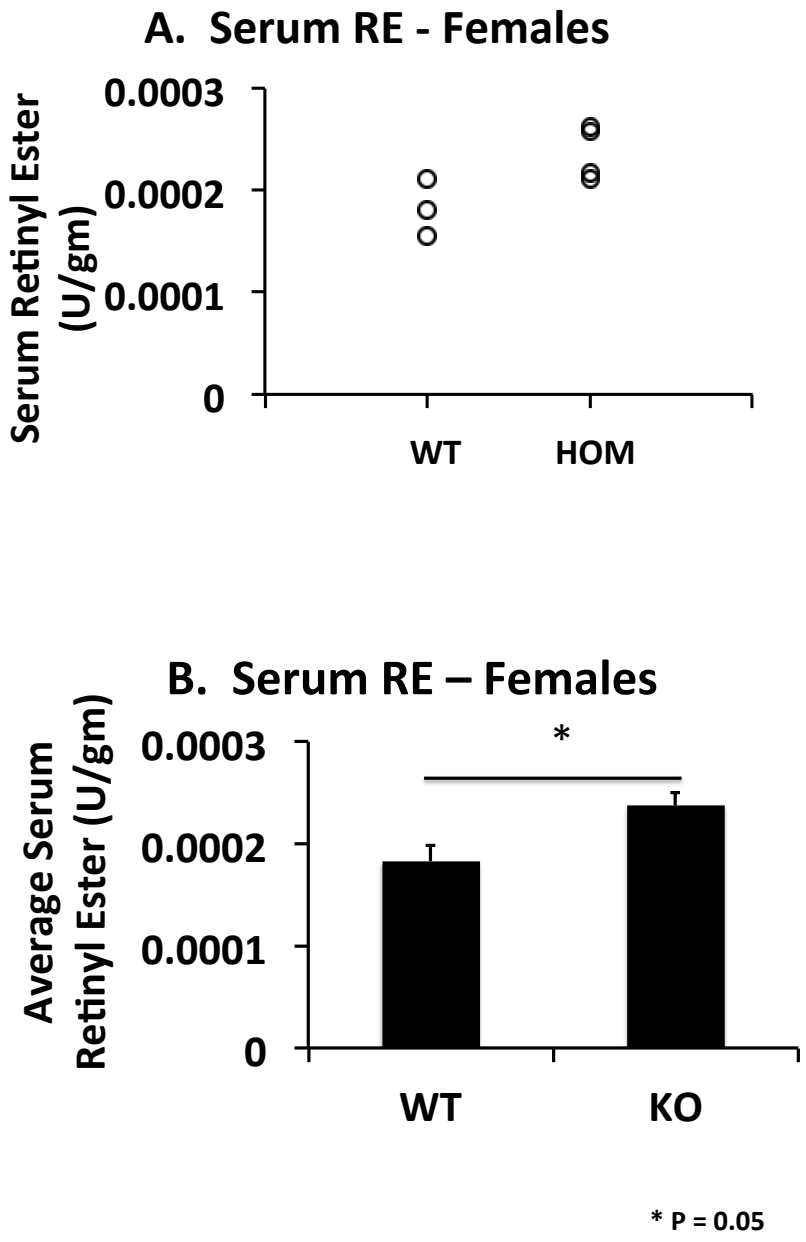


Figure 16: 7-week-old mice: serum RE – females. A. Retinyl ester levels measured in serum of WT and HOM female mice. B. Average retinyl ester levels measured in serum of WT and HOM female mice. KO mice show higher levels of RE in serum than WT.

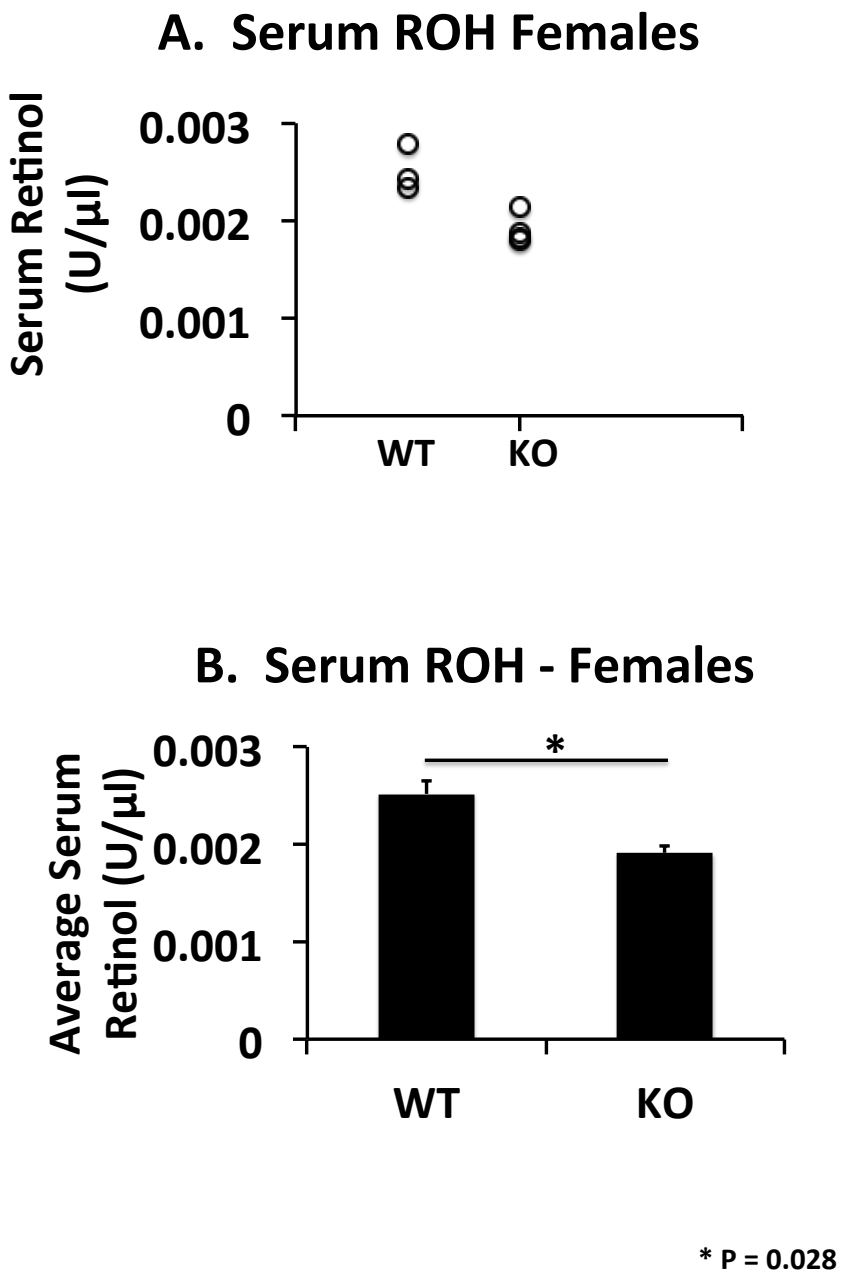


Figure 17: 7-week-old mice: serum ROH – females. A. Retinol levels measured in serum of WT and HOM female mice. B. Average retinol levels measured in serum of WT and HOM female mice. KO mice show lower levels of ROH in serum than WT.

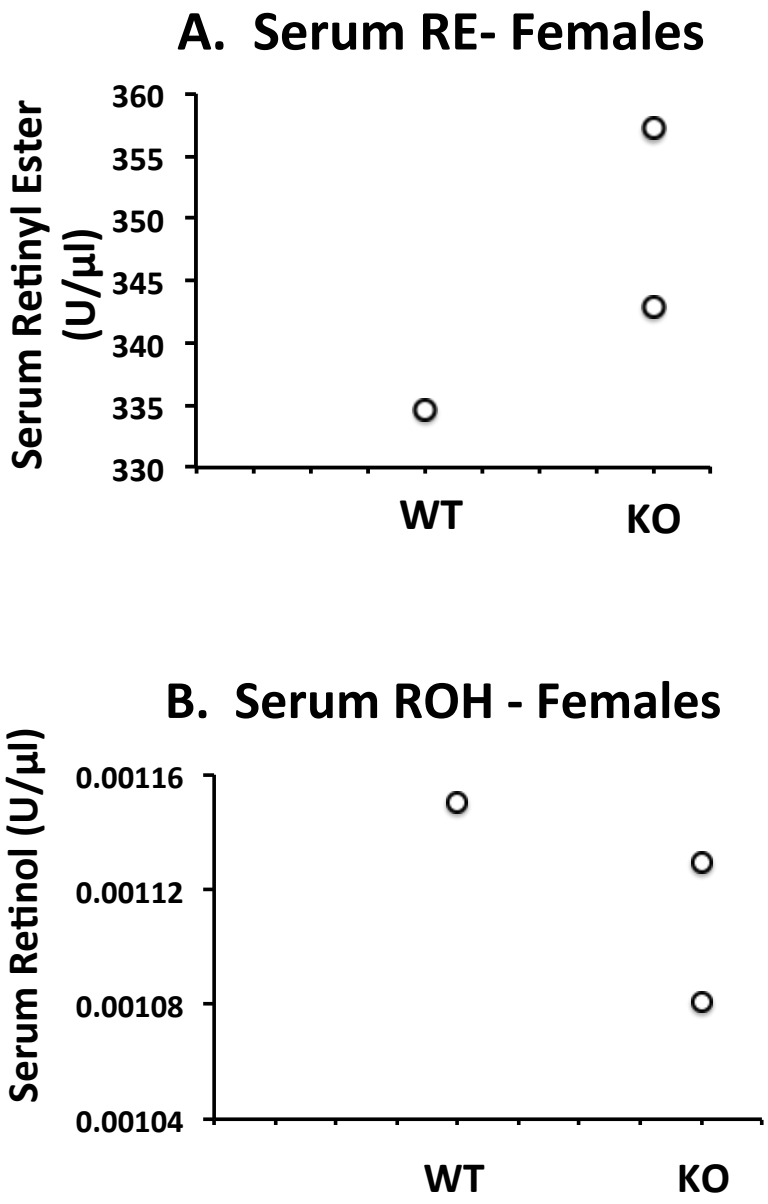


Figure 18: Adult mice: serum RE and ROH – females. A. Scatter plot showing retinyl ester levels measured in serum of WT and HOM female mice. B. Scatter plot showing retinol levels measured in serum of the same WT and HOM female mice.

DISCUSSION

This study found no discernable effect from deletion of LSH on retinoid levels in the livers or fat pads of either male or female mice, or in the serum of male mice compared to wild type control. However, a significant difference in retinoid levels was detected in the serum of female mice with LSH deletion compared to WT. Secondarily, a strong negative correlation between retinoid levels in liver and LSH gene expression was uncovered in a separate control group of WT mice (Figure 7). These findings suggest that LSH may not play a significant role in whole body retinol levels and transport, but may be involved in more targeted metabolic functions involving retinol.

LSH binds with high affinity to RBP4 and shares important homology with Stra6, the first receptor protein discovered to bind RBP4. Though RBP4 is most often described as the primary carrier protein for retinol, transport of retinol throughout the body is not solely dependent on RBP4. Retinoids from diet are packaged into chylomicrons and then secreted into the lymph system from which they enter general circulation. There, triglycerides (TG) and REs are released and hydrolyzed by the enzyme Lipoprotein Lipase (LPL) for absorption into various extra hepatic tissues. This direct delivery of retinol to tissues via chylomicrons is the best-proposed mechanism for why a deletion of RBP4 in transgenic mice causes only mild symptoms of deficiency. (1) Chylomicron remnants are ultimately taken up by the liver, the body's primary storage site for REs, (REs are also stored in adipose tissue, but to a lesser degree). Once inside the liver,

retinol can carry out any of its various functions, or be stored in the form of RE's within lipid droplets in Hepatic Stellate Cells (HSC). (16) It may also be re-packaged and shipped back out into circulation, bound to RBP4, for delivery to other tissues. (1)

Because of this RBP4-independent mechanism of retinol transport, it is possible that LSH is not necessary for maintenance of whole body retinol levels. And though it has been shown to increase retinol transport into cells *in vitro*, it may not do so *in vivo*. LSH - RBP4 binding may activate signaling of some kind and retinol may be transported only as a casual consequence of binding. LSH also may be responsible, not for whole body transport of retinol, but *targeted* transport of retinoids to specific cell types such as hepatic stellate cells, the cells that normally store RE in liver. If this is the case, mice with the LSH deletion may have liver RE levels comparable to WT, but the REs may be stored differently than in the control. More in-depth studies isolating the different hepatic cell types would be necessary to test this possibility.

An important follow up study would be one in which animals chosen were fed a Vitamin A sufficient or deficient diet in place of the standard chow diet used in this study. Because of the complexities of retinol homeostasis, specifically, the multiple pathways for its delivery to tissues, we have not been able to characterize the role of LSH from a straightforward measurement of retinol in the tissues of mice fed a diet replete with Vitamin A. The retinol intake of these animals is approximately 37 IU/g. This exceeds the Vitamin A levels deemed sufficient for mice, determined to be from 2 to 4 IU/g. (14) Ideally, animals used for any additional such study would be second generation mice fed a Vitamin A-controlled diet because even the amount of retinol pups receive through a well-fed dam's milk can provide an excess of Vitamin A. (15, 17)

A phenotype in the animals with LSH deletion may emerge in a study of second generation animals reared on a Vitamin A-controlled diet.

Another possibility that may explain the findings is that other proteins capable of acting as membrane transporters may be up-regulated in the absence of LSH to take over its function. This possibility has been investigated in rudimentary studies in our lab to measure Stra6 gene expression by taqman qPCR in the livers of mice with LSH deletion. Stra6 under normal conditions is not expressed in liver, and finding an increase in mRNA in the tissue of LSH knock out mice could explain why these mice did not show the expected retinoid – deficient phenotype. Results of these studies showed no increased gene expression of Stra6 in the livers of mice with the LSH deletion, compared to control tissue of eye and brain, known to express Stra6. There could, however, be proteins other than Stra6 responsible for taking over the transport of retinol. More studies are needed to investigate this possibility. One strategy would be to identify any proteins known to bind with fatty acid binding proteins and measure livers of LSH deletion mice for an increase in their gene expression.

Significant differences were found in the retinoid levels of female HOM and WT mice. RE found in serum is most likely to be found within chylomicrons. All mice used for this study were fed the same diet and were fasted for 16 hours before sacrifice, so any differences in remaining RE from chylomicrons could not have been due to variations in access to food, or different diets. The results from serum of female mice are intriguing, suggesting LSH may somehow play a role in efficient clearance of chylomicrons from the bloodstream. This could be tested directly by measuring RE clearance from the blood of HOM and WT mice *in vivo* after a gavage feeding of retinol in oil. Another possibility is that LSH is involved in the packaging of RE's into chylomicrons in enterocytes, where

it may play a regulatory role. A target for future studies, then, would be the intestine. Perhaps LSH is required for sequestering retinol in the enterocyte for more targeted transport.

The difference in results for serum between male and female mice is also of note, but could be due to the differences in normal growth and development between male and female mice. Male and female mice at 7 weeks of age are similar in size and weight but males begin to bulk up much faster than females at this age. Un-bred females remain lean for several more weeks than males, though they reach sexual maturity sooner (5 – 8 weeks). (18) There is some evidence that physiological mechanisms involving retinol evolved differently for males and females and the sexes may utilize retinol differently during sexual development.

Clearly, retinol is able to enter tissues without LSH - HPLC analysis has revealed similar levels in liver and fat of both HOM and WT animals. The liver ultimately takes up chylomicron remnants containing RE from diet, and it is estimated that between 65 - 75% of dietary retinol enters the liver in this way. (19) LSH may, however, play more of a regulatory role in retinol homeostasis, and though it may have the ability to transport retinol across cell membranes, it is not the only means of retinol transport. It may be similar to RBP4 which, though it is a specific carrier protein for retinol, produces only mild vitamin A deficiency when absent, which can be easily remedied through diet. Perhaps LSH is similarly involved in retinol metabolism and, like RBP4, plays a more complex role than that of a membrane transport protein.

A key strength of this study lies in our animal model. A well-studied living organism with a confirmed genetic knock out of the gene that encodes the LSH protein can provide invaluable data on the protein's role in physiology and retinol homeostasis,

but more targeted and controlled experiments need to be designed to isolate and characterize the role of LSH. The analytical method of HPLC has been sensitive enough to measure retinoids in tiny amounts of tissue and will no doubt be a useful tool in describing the function of LSH in future studies.

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